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Development of a Sensitive and Specific Assay Combining Multiplex PCR and DNA Microarray Primer Extension To Detect High-Risk Mucosal Human Papillomavirus Types

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The importance of assays for the detection and typing of human papillomaviruses (HPVs) in clinical and epidemiological studies has been well demonstrated. Several accurate methods for HPV detection and typing have been developed. However, comparative studies showed that several assays have different sensitivities for the detection of specific HPV types, particularly in the case of multiple infections. Here, we describe a novel one-shot method for the detection and typing of 19 mucosal high-risk (HR) HPV types (types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82). This assay combines two different techniques: multiplex PCR with HPV type-specific primers for amplification of viral DNA and array primer extension (APEX) for typing. This novel method has been validated with artificial mixtures of HPV DNAs and clinical samples that were already analyzed for the presence of mucosal HPV types by a different consensus PCR method, i.e., GP5+/GP6+. Our data showed a very good agreement between the results from the multiplex PCR/APEX assay and those from the GP5+/GP6+ PCR (overall rates of HPV positivity, 63.0 and 60.9%, respectively). Whereas the GP5+/GP6+ PCR was slightly more sensitive for the detection of HPV type 16 (HPV-16), multiplex PCR-APEX found a higher number of infections with HPV-33, HPV-53, and multiple HPV types. These favorable features and the high-throughput potential make our present novel assay ideal for large-scale clinical and epidemiological studies aimed at determining the spectrum of mucosal HR HPV types in cervical specimens.

Cervical cancer affects more than 400,000 women in the world each year and represents the second most common malignancy found after breast cancer (6). Cervical cancer screening is currently based on the Papanicolaou (Pap) smear, which has had a vast impact on the reduction in the incidence of this disease in developed countries. However, the sensitivities of cytological tests vary greatly according to the experience of the cytologists and the type of quality control in place (4, 12, 14, 21, 29).

Epidemiological and functional studies have clearly demonstrated that certain types of human papillomavirus (HPV) from the genus alpha of the HPV phylogenetic tree, referred to as high-risk (HR) types, are the etiological cause of cervical cancer. A recent survey of 11 case-control studies in nine countries showed that 15 different HPV types are classified as high-risk types, namely, types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82. An additional three HPV types of the same genus (types 26, 53, and 66) were classified as probable high-risk types, while several others were considered low-risk types, including HPV type 70 (HPV-70), which is phylogenetically related to the high-risk HPV types (15). The clinical value of HPV DNA testing has increasingly been recognized (10). Independent studies have shown that HPV DNA detection, when it is used as a primary screening method, has a higher sensitivity and a higher negative predictive value for the detection of preinvasive disease than the conventional Pap smear or the liquid-based cytology methods (2, 5, 22). In fact, in the United States the Food and Drug Administration has authorized the use of a commercial HPV detection test, Hybrid Capture-II (Digene), in women aged 30 years and older for primary screening, in addition to cytology and for the triage of atypical squamous cells of undetermined significance.

Constant progress in HPV typing based on PCR methods has been made over the past few years. The majority of available protocols use degenerate and/or consensus primers, followed by an additional assay that allows the identification of the specific HPV types (7, 18, 25, 27, 30). Most commonly used PCR assays amplify the L1 region (18, 28), the E1 region (26), or the E6 and E7 regions (7, 20). The use of degenerate and/or consensus primers offers the advantage of detecting a large spectrum of HPV types by a single PCR. However, they may be less efficient in detecting specific HPV types, leading to some underdetection, particularly in cases of multiple infections (3, 16, 17).

In this report, we describe the development of a reliable novel E7 PCR-based assay for the detection of a large spectrum of high-risk HPV types. The assay combines the advantages of the multiplex PCR methods, i.e., high sensitivity and the possibility to perform multiple amplifications in a single

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reaction with an array primer extension (APEX) assay. The latter method offers the benefits of Sanger dideoxy sequencing with the high-throughput potential of the microarray (8, 13, 23). Here, we also report on a comparison of the multiplex PCR/APEX and the GP5+/GP6+ PCR, followed by reverse line blotting (RLB), a well-validated assay (27).

MATERIALS AND METHODS

Development of multiplex PCR primers. Complete or partial HPV sequences were obtained from GenBank and were used for alignment of the whole E7-region gene. This region is highly divergent, and primers specific to each type were developed based on the more conserved parts of this gene. The accession numbers of the GenBank sequences that we used as references, with the corresponding HPV types given in parentheses, were X05015 (HPV-18), X74479 (HPV-45), NC_001533 (HPV-51), M74117 (HPV-35), NC_001443 (HPV-58), M62849 (HPV-39), Y14591 (HPV-68), NC_001594 (HPV-56), NC_001695 (HPV-66), NC_001635 (HPV-59), M12732 (HPV-33), NC_001592 (HPV-52), K02718 (HPV-16), J04353 (HPV-31), X74472 (HPV-26), X74482 (HPV-53), U21941 (HPV-70), X94165 (HPV-73), and AB027021 (HPV-82). Oligonucleotides were synthesized by MWG Biotech (Ebersberg, Germany) and mixed to obtain a 10× solution containing 2 μM of each primer. As shown in Table 1, the total number of primers used in the mix was 31. Two primers for the amplification of β-globin (GenBank accession number AY260740) were added to provide a positive control for the quality of the template DNA (19). Some primers are used for more than one type (e.g., HPV18R/HPV45R) due to the high similarities between the E7-region genes.

Multiplex PCR conditions. PCRs were performed with the QIAGEN multiplex PCR kit according to the instructions of the manufacturer. dUTP (Fermentas) was added to a final concentration of 50 μM, to allow PCR product fragmentation (see below). The PCR products ranged in size from 210 bp to 350 bp. The presence and the sizes of PCR products were systematically checked on agarose gels.

Generation of DNA chip. Two 5′-C-6 amino-linker-modified oligonucleotides (C-6 oligonucleotides) covering two 30-bp regions of each E7-region gene were designed, synthesized by MWG Biotech, and spotted onto silanized slides, as reported elsewhere (1a, 9). The layout of the chip is shown in Fig. 1C.

All C-6 oligonucleotides were designed in order to incorporate only uracyl (cyanine 5-ddUTP) during the extension reaction (Table 2).

APEX. By following the protocol described previously (8), the PCR products were purified, concentrated by using Millipore Y30 columns, and fragmented to facilitate the hybridization reaction with the arrayed oligonucleotides. Fragmentation was achieved by treatment of the purified PCR products with 1 U uracil *N*-glycosylase (Epicenter Technologies, Madison, WI) and 1 U shrimp alkaline phosphatase (Amersham Biosciences, Milwaukee, WI). The fragmented PCR products were added to a reaction mixture containing cyanine 5-ddUTP and cyanine 3-ddATP, -ddCTP, and -ddGTP (4 × 50 pmol); 10× buffer; and 4 U of Thermo Sequenase (Amersham Biosciences, Uppsala, Sweden) and placed onto the chip and incubated at 58°C for 10 min. After hybridization of the PCR products to the chip, the extension reaction was performed to allow incorporation of the cyanine 5-ddUTP. The slides were washed to remove the traces of the nonhybridized PCR products and the labeled dideoxynucleoside triphosphates (ddNTPs) not incorporated. The slides were imaged by use of a Genorama-003 four-color detector (Asper Biotech, Tartu, Estonia). The signal for specific HPV types in the APEX method was considered positive only if both APEX probes gave a signal in the U channel, as the probes were designed to extend only U. Although A, G, and C signals were not expected to be incorporated, ddA, ddC, and ddG were also included in the APEX reaction to allow detection of unspecific extensions and to have a further control for specificity.

The fluorescence intensities at each position were measured and converted to base calls according to the Genorama image analysis and genotyping software (Asper Biotech).

Several strategies to ensure quality control were adopted: (i) each APEX oligonucleotide was spotted in duplicate, and (ii) internal positive controls were spotted on the corners of the microarray to verify that the intensities of the four channels (A, C, U, and G) were equilibrated.

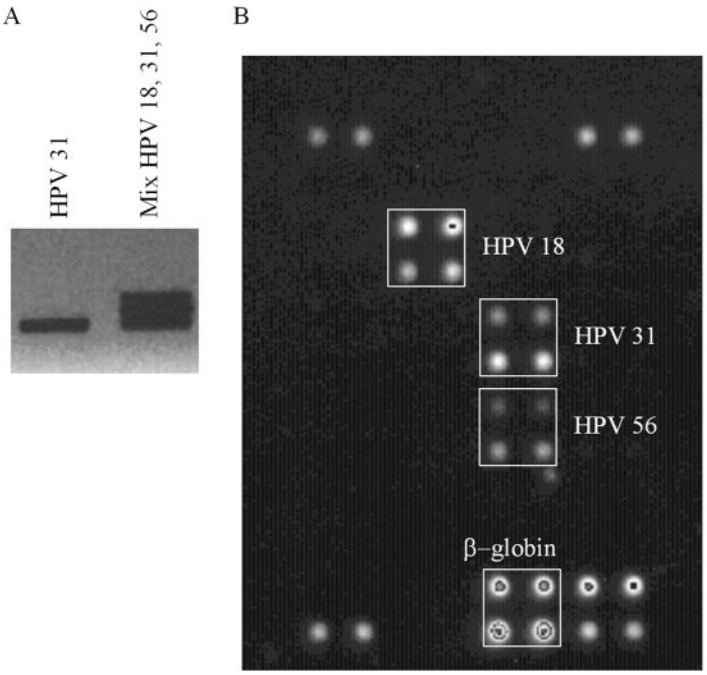
Validation of the multiplex PCR/APEX assay. For evaluation of the sensitivity and the specificity of the APEX typing method, we analyzed artificial mixtures containing cloned HPV genomes at different concentrations and, in addition, random DNA samples extracted from cervical scrapes with or without cervical abnormalities. The latter comprised 92 DNA samples that were previously typed for 37 HPV types by the HPV GP5+/GP6+ PCR reverse line blot assay (27) at the Department of Pathology, Vrije Universiteit Medical Center, Amsterdam, The Netherlands. These were liquid-based cytology samples from a group of women with abnormal or normal cytologies who participated in a population-based screening program. The DNA was extracted by using High Pure PCR

TABLE 1. Sequences of forward and reverse HPV-type specific primers and sizes of the PCR-amplified fragments[a]

HPV type	Primer sequence[b]	PCR fragment size (bp)
16	F. 5′-TGAGCAATTAAATGACAGCTCAGAG-3′ R. 5′-TGAGAACAGATGGGGCACACAAT-3′	212
18	F. 5′-GACCTTCTATGTCACGAGCAATTA-3′ R. 5′-TGCACACCACGGACACACAAAG-3′	236
26	F. 5′-CGAAATTGACCTACGCTGCTACG-3′ R. 5′-TGGCACACCAAGGACACGTCTTC-3′	239
31	F. 5′-AGCAATTACCCGACAGCTCAGAT-3′ R. 5′-GTAGAACAGTTGGGGCACACGA-3′	210
33	F. 5′-ACTGACCTAYACTGCTATGAGCAA-3′ R. 5′-TGTGCACAGSTAGGGCACACAAT-3′	229
35	F. 5′-CAACTGACCTATACTGTTATGAGC-3′ R. 5′-TGTGAACAGCCGGGGCACACTA-3′	234
39	F. 5′-TTGTATGTCACGAGCAATTAGGAG-3′ R. 5′-GACACTGTGTCGCCTGTTTGTTTA-3′	357
45	F. 5′-GACCTGTTGTGTTACGAGCAATTA-3′ R. 5′-TGCACACCACGGACACACAAAG-3′	236
51	F. 5′-GCTACGAGCAATTTGACAGCTCAG-3′ R. 5′-ATCGCCGTTGCTAGTTGTTCGCA-3′	242
52	F. 5′-ACTGACCTAYACTGCTATGAGCAA-3′ R. 5′-CAGCCGGGGCACACAACTTGTAA-3′	229
53	F. 5′-ACCTGCAATGCCATGAGCAATTGAA-3′ R. 5′-TTATCGCCTTGTTGCGCAGAGG-3′	253
56	F. 5′-ACCTACARTGCAATGAGCAATTGG-3′ R. 5′-TGATGCGCAGAGTGGGCACGTTA-3′	244
58	F. 5′-GCTATGAGCAATTATGTGACAGCT-3′ R. 5′-TGTGCACAGSTAGGGCACACAAT-3′	219
59	F. 5′-ACCTTGTGTGCTACGAGCAATTAC-3′ R. 5′-GCTGCACACAAAGGACACACAAA-3′	243
66	F. 5′-ACCTACARTGCAATGAGCAATTGG-3′ R. 5′-TGATGCGCAGAGTGGGCACGTTA-3′	244
68	F. 5′-TTGTATGTCACGAGCAATTAGGAG-3′ R. 5′-GATTACTGGGTTTCCGTTGCACAC-3′	258
70	F. 5′-CACGAGCAATTAGAAGATTCAGACA-3′ R. 5′-TTCCCGATGCACACCAGGGACA-3′	237
73	F. 5′-CTTACATGTTACGAGTCATTGGAC-3′ R. 5′-GTTTCTGGAACAGTTGGGGCAC-3′	221
82	F. 5′-GCTACGAGCAATTTGACAGCTCAG-3′ R. 5′-CATTGCCGATGTTAGTTGGTCGCA-3′	240

[a] Due the homology in the E7 gene of different HPV types, the following primers have identical sequences: HPV18R/HPV45R, HPV33F/HPV52F, HPV33R/HPV58R, HPV39F/HPV68F, HPV51F/HPV82F, HPV56F/HPV66F, HPV56R/HPV66R.

[b] F, forward; R, reverse.



C

N	HPV39_1	HPV68_1	N
A	HPV39_2	HPV68_2	C
HPV59_1	HPV18_1	HPV45_1	HPV51_1
HPV59_2	HPV18_2	HPV45_2	HPV51_2
HPV16_1	HPV35_1	HPV31_1	HPV33_1
HPV16_2	HPV35_2	HPV31_2	HPV33_2
HPV58_1	HPV52_1	HPV56_1	HPV66_1
HPV58_2	HPV52_2	HPV56_2	HPV66_2
HPV73_1	HPV82_1	HPV26_1	HPV70_1
HPV73_2	HPV82_2	HPV26_2	HPV70_2
G	HPV53_1	BGlo_1	T
N	HPV53_2	BGlo_2	N

FIG. 1. HPV typing by multiplex/APEX assay with an artificial mixture of HPV DNAs. (A) Gel image of multiplex PCR amplification products from an artificial mixture of three HPV types (types 18, 31, and 56) mixed with human genomic DNA extracted from the HPV-negative cervical cell line C33A. As a control, a PCR with only HPV-31 DNA as the template was included. (B) APEX assay of the multiplex PCR. After fragmentation of the PCR product, APEX was performed as described in the Materials and Methods. The image was obtained after excitation by a laser wavelength corresponding to cyanine 5 bound to ddUTP. The positive signals for HPV types 18, 31, and 56 and β-globin correspond to four white spots (two different oligonucleotides for each gene spotted in duplicate) and are highlighted by the white boxes. (C) Layout of the APEX chip. The oligonucleotides printed on the array are indicated by their names in the boxes. Their sequences are given in Table 2. Each oligonucleotide has been spotted in duplicate. The four corners contain a mixture of four self-elongating marker oligonucleotides that give signals in all dideoxy nucleotide channels (named N) and one self-elongation marker oligonucleotide that gives a signal only in one dideoxy nucleotide channels (A, G, C, U).

template preparation kits (Roche Applied Science, Mannheim, Germany), according to the recommendations of the manufacturer.

The purified amplicons were sequenced with the respective amplification primers on an ABI 3700 automated DNA analysis platform. The identities of the sequences obtained were verified by using Basic Local Alignment Search Tool (BLAST) analysis.

Statistical analyses. Descriptive statistics were carried out for the 92 women (panel 2) tested for HPV detection by the multiplex PCR/APEX (Lyon) and the GP5+/GP6+ PCR (Amsterdam) methods. The overall and type-specific percentages of HPV positivity were calculated for infections with single and multiple HPV types. The kappa statistic was calculated to measure the agreement between the overall rate of HPV positivity detected by the two methods beyond that expected by chance. Kappa estimates of less than 0.2 represent "poor" agreement, those between 0.2 and 0.6 indicate that the agreement can be considered from "fair" to "moderate," and kappa values more than 0.6 represent "good" or "very good" agreement (1).

RESULTS

Primer design and development of multiplex-PCR protocol. We have selected 19 different mucosal HPV types (types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82) according to their association with cervical cancers or their phylogenetic link with the high-risk types (15). After alignment of the E7-region DNA sequences of all selected HPV types, 19 pairs of primers specific for each HPV type were designed at the 3′ region of this viral gene. The sequences of all forward and reverse primers and the predicted sizes of the PCR products are shown in Table 1. As a first step, we tested by PCR the functionality of all primer pairs in single reactions using as a template the DNA of the corresponding HPV type. Each pair of primers gave a PCR product of the expected size (data not shown). Next, we tested the efficiencies of the HPV-specific primers in a multiplex PCR. All primers were mixed; and multiplex PCR was performed by using as the template serial dilutions of DNA of three randomly selected HPV types, i.e., types 16, 26, and 66 (from 1,000 to 0 copies of the viral genome). PCR products were obtained even when only 10 copies of the viral genome for each HPV type were used as template (data not shown). Thus, the presence of a large number of primers in the PCR mixture does not hamper the sensitivity of the assay.

Generation and characterization of an array primer extension assay for detection of the different HPV types. To determine the HPV types after performing a multiplex PCR, we have developed an APEX assay. Two oligonucleotides covering two distinct regions of the E7-region gene were synthesized for the 19 HPV types and were spotted in duplicate on the chip (Table 2; Fig. 1C). APEX combines two different methods, i.e., microarray and Sanger dideoxy sequencing. After PCR, the amplified DNA fragments are hybridized to the chip, which is followed by an extension reaction in the presence of ddNTPs that fluoresce differently when they are labeled. Thus, the HPV type can be confirmed by the position of the positive signal on the chip and the incorporation of the correct florescent

TABLE 2. Sequences of each of the oligonucleotides spotted on the chip

HPV type	Primer sequence
16	1. 5′-ACACACGTAGACATTCGTACTTTGGAAGACC-3′ 2. 5′-AAGCAGAACCGGACAGAGCCCATTACAATA-3′
18	1. 5′-GTAGAAAGCTCAGCAGACGACCTTCGAGCA-3′ 2. 5′-GCCGAACCACAACGTCACACAATGTTGTGTA-3′
26	1. 5′-ATTGGACTATGAACAATTTGACAGCTCAGA-3′ 2. 5′-TTGAAGCACAATGTTGTATGTGTAATAGTATAG-3′
31	1. 5′-GTGTACAGAGCACACAAGTAGATATTCGCA-3′ 2. 5′-ACCGGACACATCCAATTACAATATCGTTACCTT-3′
33	1. 5′-AGTTCGTTTATGTGTCAACAGTACAGCAAG-3′ 2. 5′-GCCACAGCTGATTACTACATTGTAACCTGT-3′
35	1. 5′-ACACACATTGACATACGTAAATTGGAAGAT-3′ 2. 5′-AGACACCTCCAATTATAATATTGTAACGTCC-3′
39	1. 5′-GCAGCTGGTAGTAGAAGCCTCACGGGATAC-3′ 2. 5′-GATGAACCACAGCGTCACACAATACAGTGT-3′
45	1. 5′-GTAGAGAGCTCGGCAGAGGACCTTAGAACAC-3′ 2. 5′-CCGAACCACAGCGTCACAAAATTTTGTGTG-3′
51	1. 5′-AACTGGCAGTGGAAAGCAGTGGAGACACCC-3′ 2. 5′-GACAGGCTACGTGTTACAGAATTGAAGCTCCG-3′
52	1. 5′-CATTCATAGCACTGCGACGGACCTTCGTAC-3′ 2. 5′-AGCCACAAGCAATTACTACATTGTGACATA-3′
53	1. 5′-ATTGAAACACAGTGTTGTAGGTGTGAGTCGTTGG-3′ 2. 5′-TGTGAGTCGTTGGTGCAGTTGGCTGTTCAGAGT-3′
56	1. 5′-CATTCAGAGTACCAAAGAGGACCTGCGTGT-3′ 2. 5′-CACGTACCTTGTTGTGAGTGTAAGTTTGTGG-3′
58	1. 5′-CACCACGGTTCGTTTGTGTATCAACAG-3′ 2. 5′-GACAGCTCAGACGAGGATGAAATAGGCT-3′
59	1. 5′-CAGCTAGTAGTAGAAACCTCGCAAGACGGA-3′ 2. 5′-ACCTGACTCCGACTCCGAGAATGAAAAAGA-3′
66	1. 5′-CATTCAGAGTACCAAAGAGGAGCTACGTGTGG-3′ 2. 5′-ACAACATAAGTGTTACCTAATTCACGTACC-3′
68	1. 5′-ACTACTAGCCAGACGGGACGAACAACAGCG-3′ 2. 5′-AACCCGACCATGCAGTTAATCACCACCAACA-3′
70	1. 5′-ACAAAATACAGTGTATGTGTTGTAAGTGTAATAC-3′ 2. 5′-TGCACTTAGTAGTAGAAGCCTCACAAGAGAACC-3′
73	1. 5′-CACGAAGTGTCAGTGCACAGTATGCCTTGCCAT-3′ 2. 5′-GACAAGCTGAACGAGAGTGTTACAGAATAGTTAC-3′
82	1. 5′-CTCGCAGTGGAAAGCAGTGGAGACAGCCTTCGCA-3′ 2. 5′-TTCAGCAAATGTTACTGGGCGACCTAAGCCTGG-3′
β-Globin	1. 5′-ATCACTTAGACCTCACCCTGTGGAGCCATACCC-3′ 2. 5′-TCCTGAGGAGAAGTCTGCCGTTACTGCCC-3′

ddNTP. To make the test simpler and applicable for two-color scanners as well, we have designed the chip oligonucleotides in such a way that, during the extension reaction, the same ddNTP (ddUTP) is incorporated in all cases. To test the specificity of the assay, we performed multiplex PCR using as the template the DNA of the individual HPV types. No false HPV-positive signals were detected by the APEX assay. In fact, hybridization of the PCR product on the chip in all cases gave a positive signal for a single HPV type (data not shown). Subsequently, we have determined the efficiency of the assay in detecting multiple HPV types. For this purpose, we have analyzed four artificial mixtures containing different HPV types: (i) HPV types 18, 31, and 56; (ii) HPV types 16, 31, 33, 39, and 68; (iii) HPV types 18, 26, 35, and 51; and (iv) HPV types 53, 56, 58, 59, and 66. In all four mixtures, the HPV DNAs were mixed with human genomic DNA extracted from the HPV-negative cervical cell line C33A. Several products were obtained by multiplex PCR, and their hybridization on the chip gave positive signals for the expected HPV types in the four artificial mixtures. The data obtained with the first mixture containing HPV types 18, 31, and 56 are shown in Fig. 1. Together, these data indicate that the multiplex PCR/APEX assay has a high sensitivity and a high specificity for detection of the mucosal high-risk HPV types.

Intermethod comparison. To validate further the novel HPV detection assay we blindly analyzed 92 DNA samples extracted from cervical scrapes collected at the Vrije Universiteit Medical Center, which were previously HPV typed by the GP5+/GP6+ PCR/RLB assay (27). For comparison studies, we took into account only the HPV types that are detectable by both assays (i.e., types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82).

Both methods detected similar percentages of HPV-positive samples (63% by multiplex PCR/APEX versus 61% by GP5+/GP6+ PCR/RLB), and the level of concordance between the two assays in identifying HPV-negative or HPV-positive samples was greater than 80% (Table 3). In addition, the GP5+/GP6+ PCR detected several low-risk HPV types (e.g., types 6, 11, 42, and 54) which were not included in the multiplex PCR/APEX assay (data not shown). Multiple infections were detected in 23 women by the multiplex PCR/APEX and in 18 women by the GP5+/GP6+ PCR/RLB (Table 3). Seven women classified as HR HPV negative according to the GP5+/GP6+ PCR/RLB result were found to be positive for infection with a single HR HPV type by the multiplex PCR/APEX assay, while only three women were negative by the multiplex PCR/APEX assay and positive by the GP5+/GP6+ PCR/RLB. Similarly, nine women infected with a single HR HPV type according to the results of GP5+/GP6+ PCR/RLB were found to have multiple infections by the multiplex PCR/APEX assay. In contrast, in only two subjects, the multiplex PCR/APEX assay detected infection with a single HPV type that was found to be infection with multiple types by the GP5+/GP6+ PCR/RLB method. Overall, more HPV types were found in multiple infections by our method ($n = 71$) than by the GP5+/GP6+ PCR/RLB assay ($n = 47$). To evaluate the specificity of our novel assay, we have selected 10 cases in which additional HPV types were detected by multiplex PCR/APEX assay in comparison to the number that were detected by the GP5+/GP6+ PCR/RLB method. After PCR, we performed DNA sequencing using HPV type-specific primers. In all cases the DNA sequencing confirmed the data obtained by the multiplex PCR/APEX assay, excluding the possibility that the higher number of HPV types detected by the novel method was due to unspecific signals (data not shown). As shown in Table 3, the greatest difference between the two assays in detecting specific HPV types was found for HPV types 16, 33, and 53. More HPV-16-positive infections were identified by the GP5+/

TABLE 3. Concordance of overall HPV positivity tested by multiplex PCR/APEX and GP5+/GP6+ PCR

HPV type and multiplex PCR/APEX result[a]	No. (%) of samples tested by GP5+/GP6+ PCR				
	HPV negative	HPV positive	Single HPV infections	Multiple HPV infections	Total
Overall					
Negative	27 (29.4)		3 (3.3)	4 (4.4)	34 (37.0)
Single	7 (7.6)		26 (28.3)	2 (2.2)	35 (38.0)
Multiple	2 (2.2)		9 (9.8)	12 (13.0)	23 (25.0)
Total	36 (39.1)		38 (41.3)	18 (19.6)	92
HPV-16					
Negative	57 (62.0)	9 (9.8)			66 (71.7)
Positive	3 (3.3)	23 (25.0)			26 (28.3)
Total	60 (65.2)	32 (34.8)			92
HPV-33					
Negative	86 (93.5)	0 (0.0)			86 (93.5)
Positive	5 (5.4)[b]	1 (1.1)			6 (6.5)
Total	91 (98.9)	1 (1.1)			92
HPV-53					
Negative	84 (91.3)	0 (0.0)			84 (91.3)
Positive	5 (5.4)	3 (3.3)			8 (8.7)
Total	89 (96.7)	3 (3.3)			92

[a] Kappa test values were as follows: overall, 0.55 (95% confidence interval, 0.41 to 0.69); HPV-16, 0.70 (95% confidence interval, 0.54 to 0.86); HPV-33, 0.27 (95% confidence interval, 0.0 to 0.69); and HPV-53, 0.52 (95% confidence interval, 0.17 to 0.88).
[b] All occurred in women infected with multiple HPV types.

GP6+ PCR/RLB assay, while HPV types 33 and 53 were better detected by the multiplex PCR/APEX assay. All five women in whom HPV-33 was detected by multiplex PCR/APEX but not by GP5+/GP6+ PCR/RLB were concurrently infected with other HPV types. Figure 2 shows the number of infections with each HPV type detected by both methods or only one method. A small number of infections with HPV types 18, 31, 59, 66, and 68 were detected by multiplex PCR/APEX and not by GP5+/GP6+ PCR/RLB, while two infections with HPV-39 were identified only by GP5+/GP6+ PCR/RLB.

The presence or absence of low-risk HPV types, as determined by the GP5+/GP6+ PCR reverse line blot method, did not affect the performance of the multiplex PCR/APEX assay. In fact, it detected similar proportions of high-risk HPV type-specific infections in samples with or without low-risk HPV types. The Fisher exact statistic for testing the differences in the proportions of infections was not significant for the most commonly detected HPV types: HPV-16 ($P = 0.56$), HPV-18 ($P = 0.99$), HPV-31 ($P = 0.74$), HPV-33 ($P = 0.33$), and HPV-53 ($P = 0.55$).

DISCUSSION

A considerable number of studies have demonstrated that detection of mucosal HPV DNAs in cervical specimens has significantly contributed to (i) identifying carcinogenic HPV types, (ii) understanding the natural history of the viral infection, and (iii) increasing the sensitivity of the screening for cervical abnormalities. Many accurate methods for HPV detection and typing have been developed in the past few decades (10). Most of these assays imply the use of degenerated and/or consensus primers that allow the detection of a large spectrum of HPV types with a single PCR. The drawback of this approach is that the various primer sets, although they are generally able to identify with a high sensitivity a broad spectrum of mucosal HPV types, have various efficiencies in detecting multiple infections (3, 16, 17). Intermethod comparisons clearly showed that the use of only one detection method could easily lead to an underestimation of the overall prevalence of HPV DNAs in clinical specimens (11, 24). Therefore, the use of more than one assay would be the preferred means for the most reliable assessment of the prevalence of the complete spectrum of relevant HPV types in epidemiological studies.

Here, we describe a novel multiplex PCR-based method with a panel of HPV type-specific primers. This assay detects a wide spectrum of mucosal high-risk HPV types with a high sensitivity by a single PCR and combines the advantages of PCR-based methods that use degenerated/consensus primers and type-specific primers. In addition, in order to identify the HPV types amplified by the multiplex PCR, we have developed a chip assay based on APEX. A comparative analysis between our method and the well-validated PCR-based assay, the GP5+/GP6+ PCR, followed by RLB, showed that the multiplex PCR/APEX protocols led to the identification of a higher number of infections with multiple HPV types. In this study, several HPV types were found more frequently by the novel method than by the GP5+/GP6+ PCR/RLB assay, especially in women infected with multiple HPV types. These results are in agreement with the idea that in the case of multiple infections, different HPV types will not compete for the same primer set, which is in contrast to case for the GP5+/GP6+ PCR method, which uses only one primer set.

The limitation of the current version of our assay is the lower efficiency of detection of HPV-16 than that by the GP5+/GP6+ PCR/RLB protocol. This phenomenon may be explained by possible natural variations within HPV-16 E7-re-

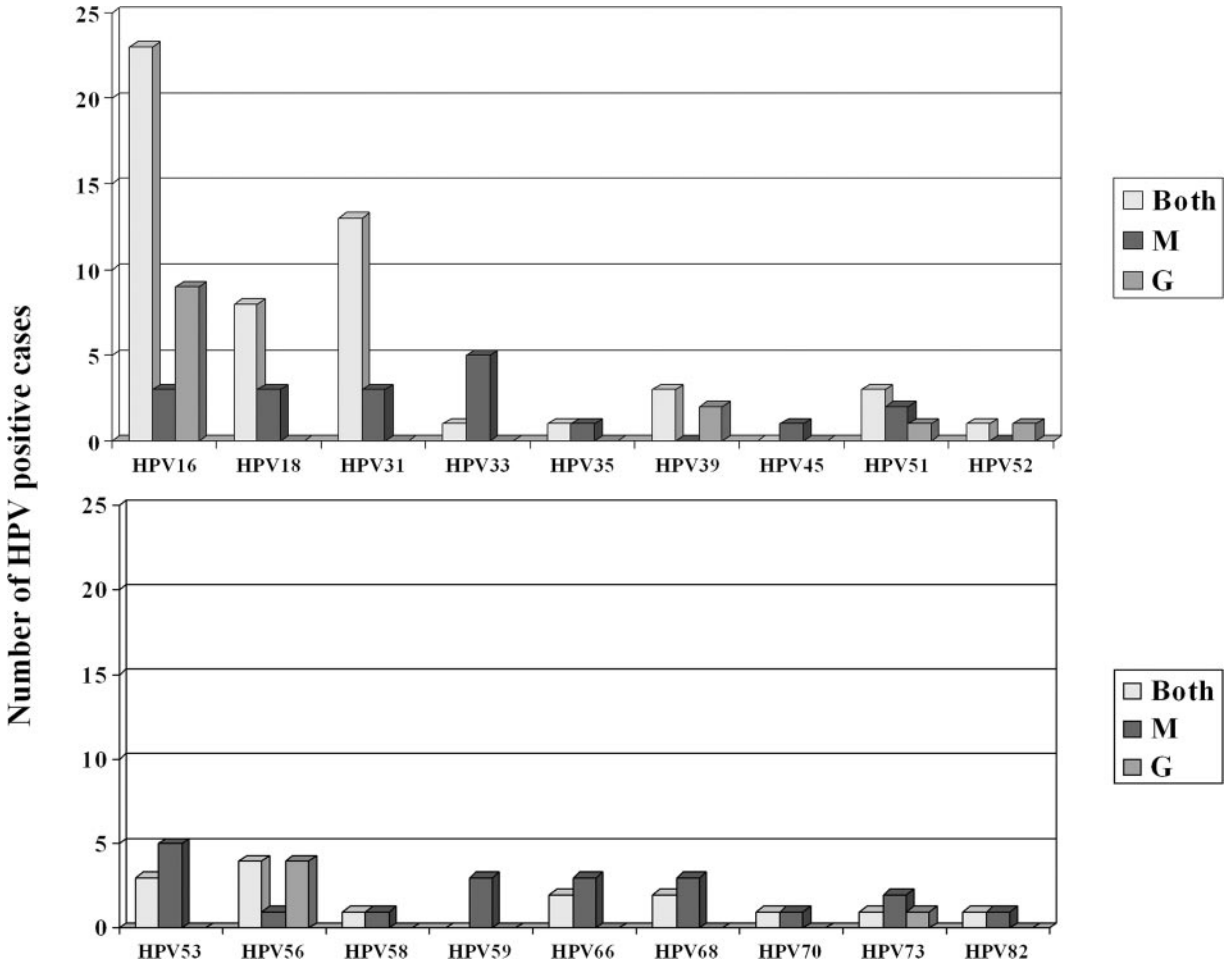


FIG. 2. Frequency of detection of different HPV types by the multiplex PCR/APEX (M) and the GP5+/GP6+ PCR/RLB (G) methods in 92 cervical scrapes of Dutch women.

gion genes. In fact, a natural variant with a variation in the region selected for the forward PCR primer has been reported (31). In addition, we are currently investigating whether this apparent reduced sensitivity of the multiplex PCR/APEX method is due to the primers used in the PCR or on the chip. Due to the general features of the multiplex PCR/APEX assay and its high degrees of versatility and flexibility, the sensitivity of the assay for detection of these HPV types can easily be improved by including new or modified oligonucleotides in the multiplex PCR and/or on the chip. Another advantage of our assay is that the PCR amplifies a part of the E7-region gene that is retained in all cancer cells. In contrast, many HPV detection protocols use PCR primers that anneal in the L1 gene, which may be lost or deleted during the integration of the viral DNA in the host genome.

In conclusion, this study describes a novel assay for detection of mucosal high-risk HPV types with a high sensitivity and a high specificity for the detection of several mucosal high-risk HPV types in women infected with single and multiple HPV types. This method may be a very valuable tool for epidemiological studies that aim to determine the distributions of various HPV types in different areas of the world.

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